

Noninvasive Methods for Measuring DNA Alkylation in Experimental Animals and Humans

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Alkylpurines are liberated from alkylated DNA by glycosylase repair enzymes and, in most cases, excreted in urine without further metabolism. This phenomenon forms the basis of noninvasive methods to measure DNA alkylation *in vivo*. In the case of methyl adducts, such as 7-methylguanine (7-MeGua), natural backgrounds exist due to RNA turnover. However, deuterated (d_5) methylating agents or precursors give rise to d_5 -7-MeGua and d_5 -3-methyladenine (3-MeAde), which can be readily quantitated using gas chromatography-mass spectrometry (GC-MS). A deuterated probe drug, such as d_5 -aminopyrine, can be used to measure endogenous nitrosation levels in experimental animals. In contrast, for higher alkyl homologues of alkylpurines, natural backgrounds are low or nonexistent and can be directly measured by GC-MS using stable isotope labeled internal standards. For example, increased levels of urinary 3-ethyladenine were observed in cigarette smokers. Due to recent advances in analytical methodology, notably immunoaffinity cleanup of urine, measurements of excreted DNA adducts can be used in studies in human populations exposed to low levels of alkylating carcinogens.

Introduction

Alkylating carcinogens react with DNA at most, if not all, of the nucleophilic nitrogen and oxygen centers. The major sites of reaction are at the N7 and O⁶ of guanine, N3 of adenine, and O⁻ of the phosphate residues (1). In recent years, a number of efficient repair systems have been discovered that can remove various carcinogen-DNA adducts (2). The glycosylases liberate the alkylated purine base, leaving an apurinic site that is subsequently repaired. In contrast, alkyltransferases mediate the transfer of the alkyl group to a receptor cysteine residue in the repair protein, thus restoring the original base.

In many cases, alkylpurines that are liberated by glycosylases are excreted intact in urine, as these adducts do not appear to be substrates for catabolic enzymes [reviewed by Shuker and Farmer (37)]. These observations suggested that it ought to be possible to determine the level of modification of DNA at all sites by measuring alkylpurines in urine. Interestingly, Craddock and Magee

(4) found that rats whose DNA had been specifically labeled with ^{14}C and which were subsequently treated with ^3H -labeled nitrosodimethylamine (NDMA), excreted [^3H , ^{14}C]-7-methylguanine (7-MeGua), thus demonstrating a direct link between DNA methylation and urinary excretion of methylated adducts. Recently, Gombar et al., (5) showed that [^{14}C]-7-MeGua was excreted by rats that had been treated with [^{14}C]aminopyrine (AP) and nitrite, a combination known to result in endogenous synthesis of [^{14}C]NDMA. In addition, it was shown that there was an excellent correlation between DNA methylation in liver (target organ) and urinary excretion of 7-MeGua and that the urinary adduct was readily detectable at low doses of AP and nitrite where DNA methylation was undetectable.

In view of the natural occurrence of certain urinary methylpurines as a result of the turnover of RNA, in which certain methylated nucleosides are present at relatively high levels and are crucial to the normal functioning of RNA [for example, 7-methylguanosine is present in the end-capping region of tRNAs (6)], most studies on methylation have been carried out using radioactively labeled carcinogens. Although this is clearly useful in experimental studies, it is impracticable and ethically unacceptable (other than in exceptional circumstances such as in the development of new drugs) to use radiolabeled compounds in human volunteers. For this reason, a number of groups have used stable isotope-labeled methylating agents and

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precursors, and this area is reviewed here. The problems outlined above are not as great when alkylating exposures other than methylation, which is a frequently encountered biochemical process, are studied. For example, adducts from aflatoxin B₁ and benzo[*a*]pyrene are sufficiently characteristic that it is extremely unlikely that they would be formed by any other route than by exposure to the carcinogen. Recent results on the development of noninvasive methods to measure DNA alkylation *in vivo* are presented in this paper.

Excretion of d₃-Methyl Adducts after Administration of d₃-Methylating Agents or Precursors

Stable isotope labelling (typically with ²H[d], ¹³C, or ¹⁵N) of alkylating carcinogens or precursors not only allows metabolic pathways to be studied using mass spectrometry but also offers the potential for use in human studies. The transfer of intact d₃-methyl groups from hexadeuterated NDMA to N7 of guanine in DNA and RNA *in vivo* was demonstrated first by Lijinsky et al. [(7) Fig. 1]. Similar reactions were shown for N-methyl-N'-nitro-N-nitroso-guanidine (8) and 5-(3-methyl-1-triazeno)-imidazo-4-carboxamide (9).

More recently, the excretion of d₃-7-MeGua was used to study the *in vivo* nitrosation and alkylating activity of potentially nitrosatable drugs containing N-d₃-methyl functions. Thus, coadministration of d₆-aminopyrine (d₆-AP) and nitrite to rats resulted in dose-dependent excretion of d₃-7-MeGua, whereas administration of d₆-AP did not give detectable levels of deuterated adduct (10). In rats, Farmer et al. (11) found that d₃-7-MeGua was excreted in urine after treatment with d₃-methylcyclohexylnitrosamine (d₃-MNCA), which is the nitrosation product of the deuterated analogue of the drug bromhexine. However, in human volunteers who received a clinically used dose of d₃-bromhexine, no excretion of d₃-7-MeGua was observed,

which suggests that endogenous nitrosation of this drug, if it happens at all, occurs to a very low extent (12).

7-MeGua is present in urine as a result of RNA turnover at levels that interfere with the determination of low levels of d₃-7-MeGua. This interference is due to the presence of natural isotopes (notably, ¹³C) that contribute to the M⁺ + 3 peak, and this sets a practical limit of detection that is much higher than could be obtained by the mass spectrometric methods currently available. This problem can be circumvented by choosing an excreted methyl adduct for which the natural urinary background is low or nonexistent. This criterion is met by 3-methyladenine (3-MeAde), which is present in human and rat urine at 0.14% and 0.12%, respectively, of the levels of 7-MeGua (13). A similar dose-response relationship to that described above for urinary d₃-7-MeGua was seen for the excretion of d₃-3-MeAde in rats after coadministration of combinations of d₆-AP and nitrite (13). These results, in combination with recent improvements in analytical methodology using immunoaffinity purification of 3-MeAde (14), suggest that d₆-AP could be a useful probe drug for studies on endogenous nitrosation in experimental animals (AP cannot be used as a probe drug in humans due to its toxicity). Studies are currently underway in our laboratory to evaluate the role of parasitic infections in endogenous nitrosation using d₆-AP in experimental models.

Urinary 3-Alkyladenines as Markers of DNA Alkylation in Humans.

As described in the previous section, the major DNA adducts formed from methylating agents are repaired by glycosylases, resulting in the formation of methylpurines, which are subsequently excreted in urine. These observations appear to be applicable to higher alkyl homologues and are summarized in Figure 2. The rationale for using 3-alkyladenines (3-alkAde), in particular, as noninvasive markers for DNA alkylation *in vivo* has been described recently (13). Immunoaffinity columns were prepared

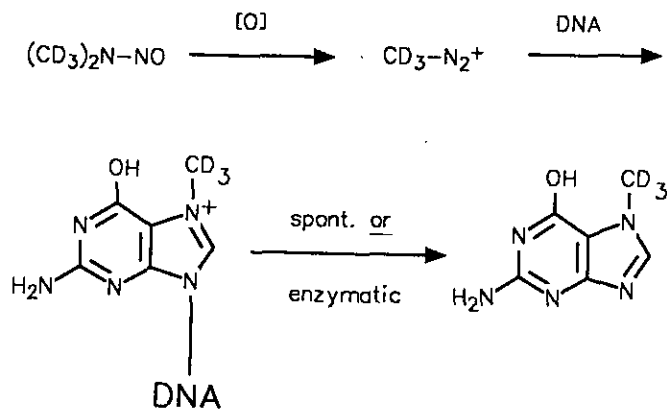


FIGURE 1. Trideutero(d₃)methylation of guanine in DNA by d₆-nitrosodimethylamine. Note the conservation of the three deuterium atoms via the intermediate d₃-methyl diazonium ion.

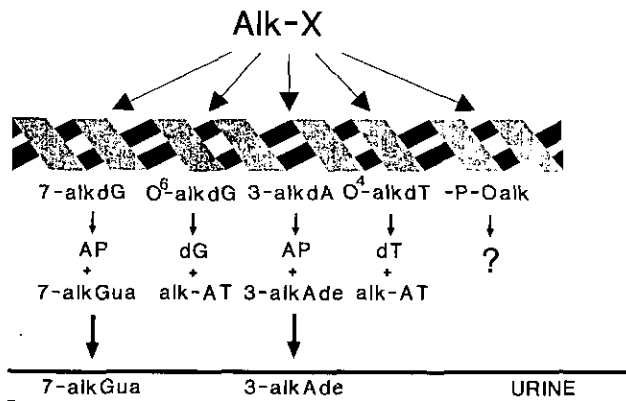


FIGURE 2. Summary of the formation and fate of the major DNA adducts derived from alkylating carcinogens (Alk-X). AP, apurinic sites; alk-AT, alkylated alkyltransferase protein.

using a monoclonal antibody that cross-reacts with a number of different 3-alkAde (alkyl = C₁-C₇) and were used to selectively extract these adducts from urine before GC-MS analysis (15). Using this methodology it was demonstrated that a) 3-alkAde such as 3-MeAde, 3-EtAde, and 3-HOEtAde were excreted essentially unchanged in humans (3-BzAde, in contrast, appeared to undergo extensive metabolism) and b) "background" levels of 3-alkAde, other than 3-MeAde, were progressively much less dependent on diet as the molecular weight of the alkyl group increased (which is probably related to the decreasing likelihood of natural sources of higher molecular weight 3-alkAde compared to 3-MeAde).

The low or nonexistent backgrounds of some 3-alkAde in human urine has allowed studies to be carried out in humans exposed to environmental carcinogens. For example, 3-ethyladenine is excreted in urine of cigarette smokers at readily detectable levels, which correlate very well with tobacco consumption (16). Studies currently underway in our laboratory also include the measurement of characteristic 3-alkAde in urine of cancer patients treated with certain chloroethylnitrosoureas.

Conclusions

In this brief summary of recent progress on the development and use of noninvasive methods for measuring exposure to alkylating carcinogens, two main approaches have been described: the use of deuterium-labeled methylating carcinogens or precursors in studies of DNA methylation *in vivo* and the measurement of urinary 3-alkAde as markers of DNA alkylation. These methods have benefited from recent advances in analytical methodology, notably, the use of immunoaffinity purification of DNA adducts from urine in combination with low resolution gas chromatography-mass spectrometry, which enables their use in studies in human populations exposed to low levels of alkylating carcinogens.

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